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Synthesis and Characterisation of Steroid Metabolites for Use as Analytical Reference Materials

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SUMMARY

The availability of the appropriate reference materials (RMs) and certified reference materials (CRMs) is a fundamental requirement for harmonising methodology for the detection of anabolic steroid abuse. This paper describes the approach we are developing to synthesise, characterise and certify these materials in accordance with the established ISO-REMCO Guidelines for Reference Material production and reports our progress to date.

INTRODUCTION

A submission to the Lausanne IOC conference on doping in sport cited the importance of the provision of anabolic steroid RMs and CRMs for the harmonisation and validation of testing methodology world-wide[1]. This will be particularly important if the proposed ISO protocol for sports drug testing [2] is adopted. In the lead up to the Sydney 2000 Olympics we are undertaking a programme aimed at the preparation of a collection of anabolic steroid RMs and CRMs. The Pure Substance Reference Material team of the National Analytical Reference Laboratory, in close collaboration with the Australian Sports Drug Testing Laboratory is undertaking the programme. Before proceeding further, it is important to clarify the meaning of the terms Reference Material and Certified Reference Material, based on the definitions given in ISO Guide 30[3].

Reference Material (RM): "A material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method or for assigning values to materials."
These materials are variously referred to as working reference materials, check materials, quality control standards or secondary working standards. They are intended for day-to-day use in monitoring instrument performance and detecting method drift and for routine calibration and assessment of the performance of an analytical system.

Certified Reference Material (CRM): "A material or substance accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realisation of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty statement at a stated level of confidence."
Traceability is defined as the "property of the result of a measurement or the value of a standard whereby it can be related, with a stated uncertainty, to stated references, through an unbroken chain of comparisons" [3].
Certified Reference Materials are intended as analytical benchmarks that can be used for

- method development and validation
- verification of the correct application of standardised procedures
- calibration and verification of a measurement process [4].

Most chemicals from commercial sources are well-characterised homogeneous compounds. They are usually provided with an analytical report, often described as a "certificate of analysis". However this does not mean they can be described as CRMs. The certification process requires a detailed and critical evaluation of the data obtained in the characterisation stage with the objective of establishing a traceable value for the key properties of the material. The critical properties of pure single substance organic reference materials are their identity and their chemical purity and, for deuterium-labelled compounds, their isotopic purity. To establish the traceability of a CRM its property values must be produced with an accompanying uncertainty estimate that establishes the confidence limit that can be placed on the property value. In addition

the producer of the CRM should be operating using a transparent quality system that covers all aspects of the production process [5].

DISCUSSION

An overview of the procedure we have developed for the production and certification of reference materials is shown in Figure 1. The implementation of this protocol will be outlined below where its application in the production of steroid metabolite RMs is described.

The first stage of the process is the identification of end-user needs. There are two primary analytical goals in anabolic steroid screening, each having different RM needs. One goal is to detect the external application of endogenous steroids, in particular testosterone, by detection of significant deviations in the endogenous steroid profile [6]. The optimal reference compounds for this assay are deuterated steroid conjugates. These materials have the same chemical reactivity and chromatographic properties as the unlabelled steroid but can be clearly differentiated from the endogenous material by GCMS. The addition of these compounds as internal standards and surrogates allows for monitoring of method recovery and assay bias, accurate quantification of endogenous steroid levels and the rapid detection of interference within the complex assay matrix. The rationale for the use of these compounds, initial synthetic studies in this area and their applications in steroid analysis have been described elsewhere[7-10].

The goals of our program in this area are:

- synthesis of multigram amounts of each target steroid, both unlabelled and deuterated
- synthesis of gram amounts of key glucuronide and sulphate conjugates
- characterisation of the materials as RMs and/or CRMs

Forty-four compounds, comprised of ten deuterated steroids, their unlabelled analogues and twenty-four key conjugates (glucuronides and sulphates) were targeted for production. The synthesis of the labelled target analytes (marked * in Fig. 2) have been completed. The syntheses we developed for d_3-testosterone (Fig. 3), d_3-epitestosterone (Fig. 4) and d_4-androsterone (Fig. 5) differ significantly from published procedures and are outlined schematically. These synthetic procedures will be described more fully in a subsequent publication. In the other cases minor variations on established synthetic routes were used [7, 11]. In addition all the sulphates of the labelled steroids and the glucuronides of d_3-testosterone, d_3-5α-DHT and d_4-androsterone have been prepared. The synthesis of d_3-epitestosterone glucuronide is also at an advanced stage. With

one exception the perdeuterated material comprises more than 85% of the total deuterated species and negligible amounts of the unlabelled compounds are present. The deuteration levels are summarised in Table 1. When the synthesis of d_4-androsterone was scaled up to a multi-gram level, the deuteration level obtained was lower than expected and than was observed in smaller scale preparations, probably as a result of hydrogen/deuterium exchange during the oxidation step to the dione intermediate. However the compound is still suitable for use in its intended application.

The second goal is the detection of doping with synthetic anabolic steroids. The problem in this case is one of low-level detection. Unlabelled reference materials are required to calibrate apparatus, establish detection thresholds and to act as positive controls in confirmation assays. The target materials selected were the key marker metabolites identified in the literature for specific synthetic anabolics [12].

The goals of our program in this area are:

- synthesis of multigram amounts of each (unlabelled) target steroid
- synthesis of gram amounts of the key glucuronide conjugates
- characterisation of the materials as RMs and/or CRMs

The second objective is an ambitious one, given that no syntheses of these glucuronides have been reported in the chemical literature and in particular the glucuronides of 17α-methyl anabolic steroids are expected to be unstable. In total fifty-three compounds, comprised of thirty-two free steroids and twenty-one glucuronides are under investigation. The preparation of these materials was sub-contracted to two external laboratories and our role has been to co-ordinate the synthetic effort and to characterise and confirm the identity of the materials as they are made available.

As the compounds are prepared, the confirmation of their qualitative identification and characterisation of their chemical and isotopic purity is undertaken in accordance with published guidelines [13, 14]. Use is made of various independent sources of structural information and identification. Only if all these investigations are consistent with the anticipated structure can the identification be made with confidence. The minimum characterisation data we stipulate for our materials are:

i. GC-MS, IR, ^{1}H and ^{13}C NMR spectra (and ^{2}H NMR for deuterates)

ii. GC-FID and/or HPLC chromatogram

iii. TLC properties

iv. Melting point

v. C, H, N microanalytical data.

If a well-characterised sample of the compound is available our material and the comparison sample are compared for the congruence of their chromatographic properties. If the silylated derivatives of the two materials co-elute from the GC and their mass spectra are identical this provides very strong evidence of their qualitative equivalence. For isotopically labelled steroids the equivalence of its retention time with the unlabelled endogenous steroid is confirmed. Co-elution of a mixture under HPLC or TLC analysis provides further confirmatory information.

Once the identification of the material is established satisfactorily the purity and homogeneity of the bulk material is measured. This is determined by a minimum of two chromatographic techniques using a statistically valid sampling protocol. GC-FID, GC/LC-MS, HPLC with UV or RI detection, elemental analysis and ^{1}H NMR are all used where appropriate and a GCMS scan in SIM mode is used to determine deuteration levels. Thermogravimetric is used to assay both for levels of volatile impurities and non-volatile residues. The important principle is that a number of complimentary, independent methods are used to assess the purity of the substance, in order to obtain the most accurate determination possible. For homogeneity testing the absolute accuracy of the method used is not as important, but it must have a high degree of precision so that any significant variations in the composition of the bulk material will be detected. We test a minimum of ten samples taken from throughout the bulk material.

We are developing and validating procedures to derive an uncertainty budget for the purity values obtained in this process. Determining the confidence level for the compound's purity estimation is the final step in establishing traceability for the material, one of the key requirements for the provision of materials as CRMs. Accelerated stability trials are underway to establish the shelf life of the materials. All RMs are stored and labelled appropriately, and analysis reports and certificates are produced in compliance with the recommendations in the relevant ISO Guide [15].

In the final step the data is submitted to a review panel which contains independent experts from outside our organisation. They assess all aspects of the preparation, characterisation and certification process prior to compounds being made available as fully characterised RMs and/or

CRMs. A RM or CRM cannot be issued until the review panel has confirmed our characterisation and assignment of its property values.

The compounds identified for production and the current status of the synthesis and characterisation process are summarised for the endogenous steroid metabolites in Table [2] and for the synthetic steroid metabolites in Table [3]. The target date for completion of the project is June 2000. Ten unlabelled steroids, ten deuterated congeners and fifteen conjugates have been prepared and characterised to date, and nine have been taken through the review process and are available as Reference Materials. For the synthetic steroids twenty four of the target fifty three compounds have been synthesised to date (July 1999) and thirteen have been taken through the review process and are also available for use as Reference Materials.

CONCLUSION

We have made significant progress in preparing a collection of anabolic steroids metabolites that are fit-for-purpose for the current requirements of sports drug testing laboratories, in the process developing a general protocol that satisfies the ISO requirements for Reference Material production. Ongoing work will expand the range of available compounds and will allow for their provision where relevant as Certified Reference Materials.

REFERENCES

1. Segura, J., Reliability in dope testing: The system of IOC-Accredited Laboratories. Submission to the World Conference on Doping in Sport, Lausanne, Switzerland, 1999.
2. Mendoza, J. and Andersen, R. Doping Control in Sport - harmonizing procedures and practices, ISO Bulletin (July 1999), 6-11.
3. ISO Guide 30, (1992): Terms and definitions used in connection with reference materials.
4. ISO Guide 33, (1989): Uses of Certified Reference Materials.
5. ISO Guide 34, (1996): Quality system requirements for reference materials producers.
6. Ayotte, C., Goudreault, D. and Charlesbois, A., Testing for natural and synthetic anabolic agents in human urine. *J. Chromatogr. B.* 1996, **687**, 3-25.
7. Schänzer, W and Donike, M., Synthesis of Deuterated Steroids for GC/MS Quantification of Endogenous Steroids. In: *Proceedings of the 12th Cologne Workshop on Dope Analysis*, Sport und Buch Strauß Köln, (1995), 93-112.

8. Nolteernsting, E., Geyer, H., Mareck-Engelke, U., Schänzer, W and Donike, M., Standardisation of the T/E Determination by Deuterated Internal Standards. In: *Proceedings of the 12th Cologne Workshop on Dope Analysis*, Sport und Buch Strauß Köln, (1995), 113-120.
9. Sanaullah and Bowers, L., Facile Synthesis of [16,16,17-$^2H_3$]-testosterone and -epitestosterone and their Glucuronides and Sulfates. *J. Steroid Biochem. Molec. Biol.*, 1996, **58**, 225-234.
10. Geyer, H., Schänzer, W., Mareck-Engelke, U., Nolteernsting, E. and Opfermann, G., Screening Procedure for Anabolic Steroids - The Control of the Hydrolysis with Deuterated Androsterone Glucuronide and Studies with Direct Hydrolysis. In: *Proceedings of the 15th Cologne Workshop on Dope Analysis*, Sport und Buch Strauß Köln, (1998), 99-101.
11. Wudy, S., Synthetic procedures for the preparation of deuterium-labeled analogs of naturally occurring steroids. *Steroids*, 1990, **55**, 463-471.
12. Schänzer, W. and Donike, M., Metabolism of anabolic steroids in man: synthesis and use of reference substances for identification of anabolic steroid metabolites. *Analytica Chimica Acta*, 1993, **275**, 23-48.
13. ISO Guide 35, (1989): Certification of reference materials - General and statistical principles.
14. Guidelines for the production and certification of BCR reference materials (1994).
15. ISO Guide 31, (1996): Contents of certificates of reference materials.

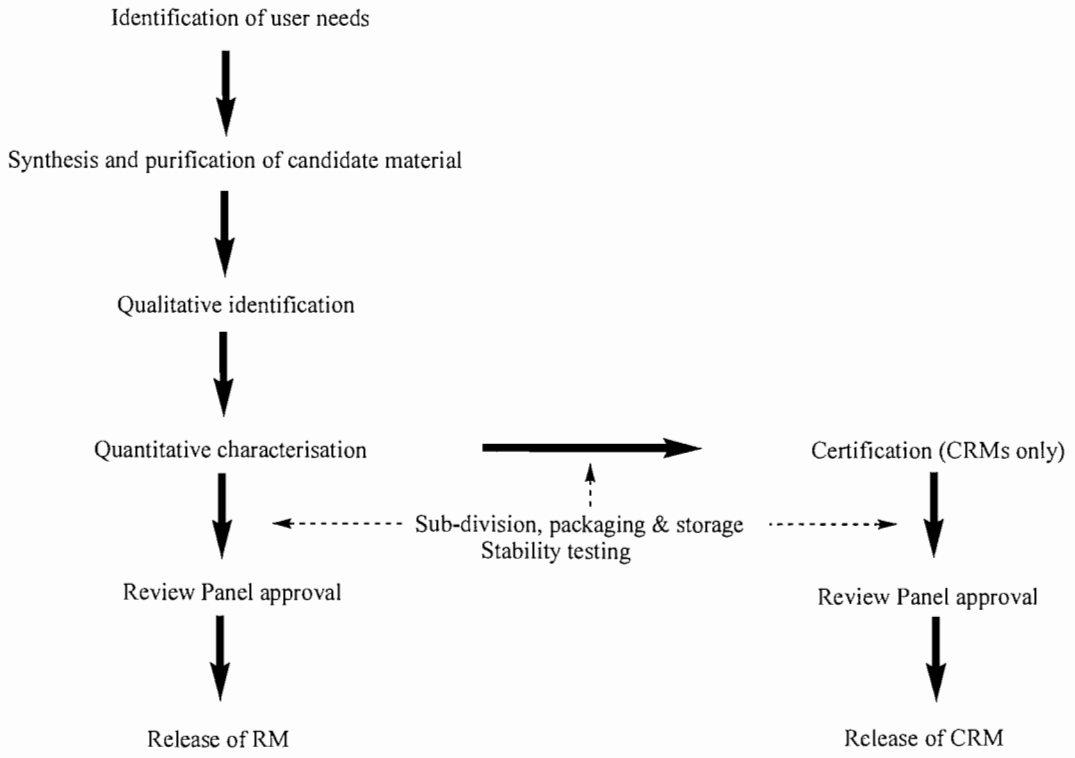

Fig. 1: Overview of the RM/CRM Production Protocol

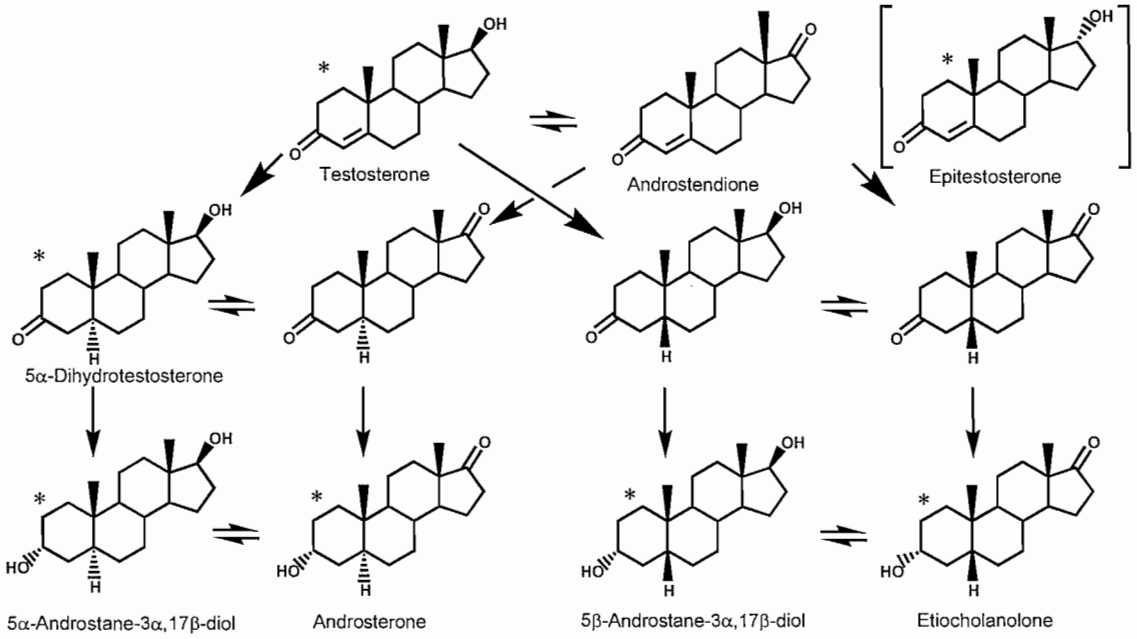

Fig. 2: Metabolic pathway for testosterone. Compounds marked * have been synthesised as deuterated reference materials

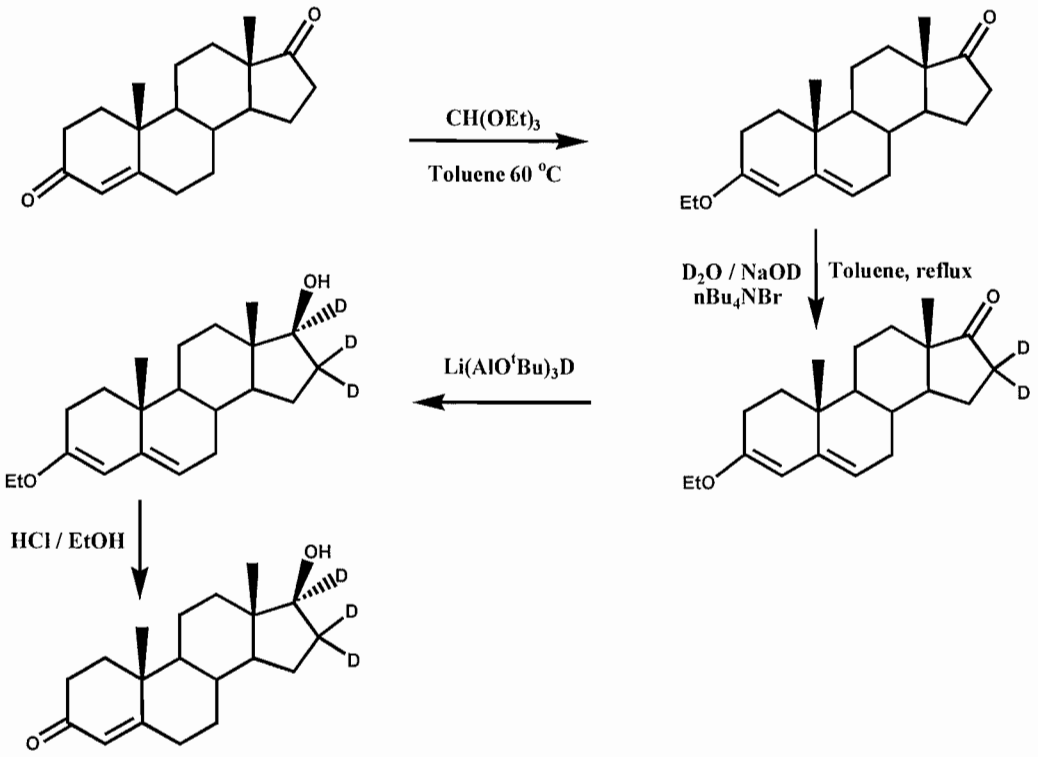

Fig. 3: Synthesis of [16,16,17-$^2H_3$]-Testosterone

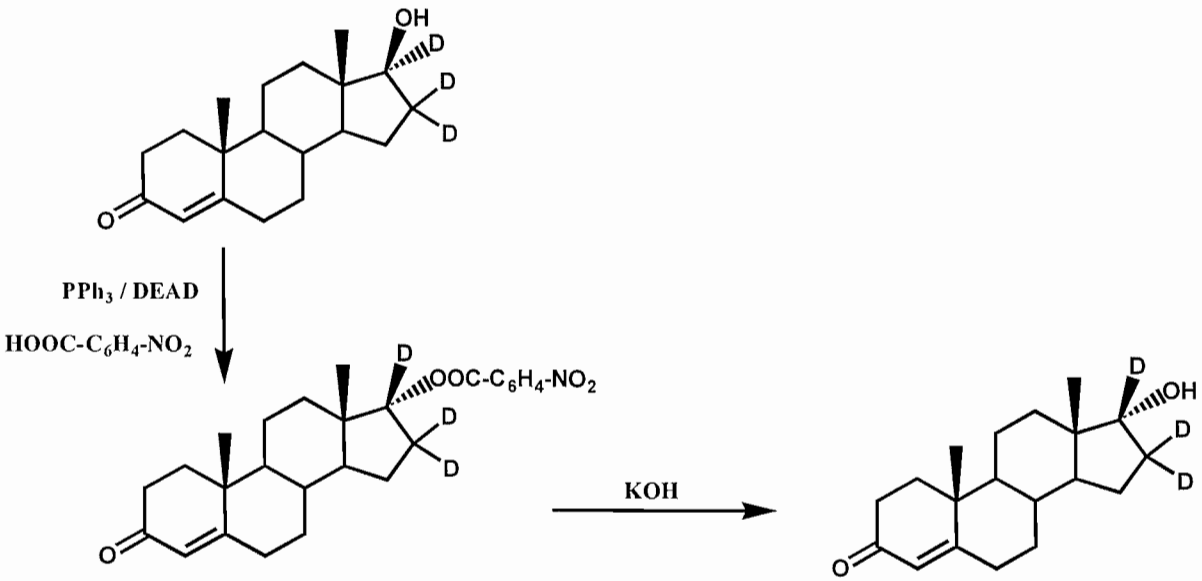

Fig. 4: Synthesis of [16,16,17-$^2H_3$]-Epitestosterone

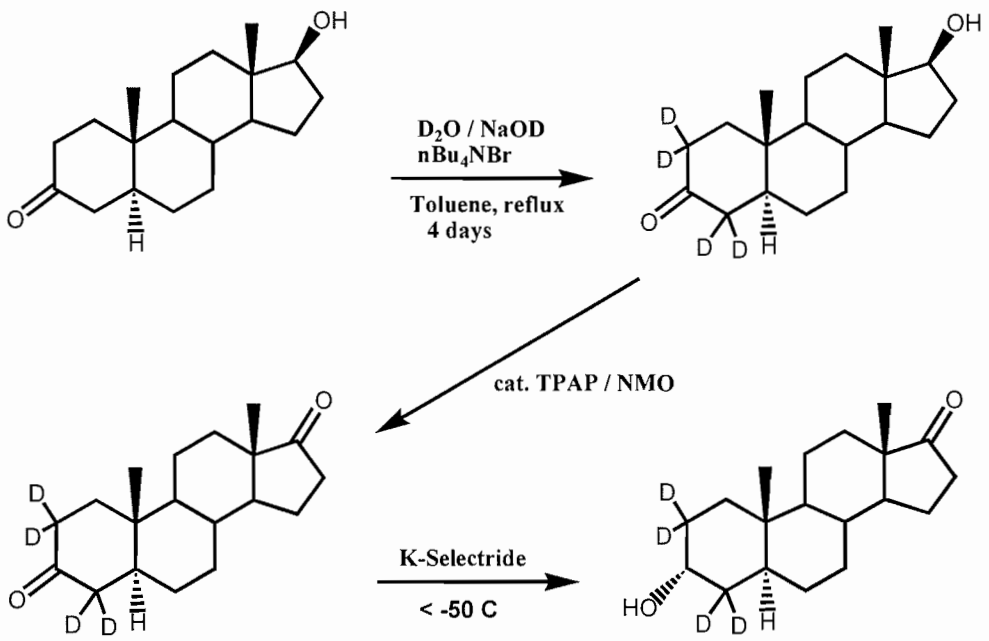

Fig. 5: Synthesis of 2,2,4,4-$^2H_4$-Androsterone

Steroid	Deuterium content* (%)					
	$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$	$^2H_5$
(16,16,17-$^2H_3$)-Testosterone	0	1	8	**91**		
(16,16,17-$^2H_3$)-Epitestosterone	0	1	6	**93**		
(16,16,17-$^2H_3$)-5α-DHT	0	1	5	**94**		
(2,2,4,4-$^2H_4$)-Androsterone	1	1	2	15	**81**	
(2,2,3β,4,4-$^2H_5$)-Etiocholanolone	0	0	0	1	6	**93**
(16,16,17-$^2H_3$)-5α-Androstane-3α,17β-diol	0	1	4	**95**		
(2,2,3β,4,4-$^2H_5$)-5β-Androstane-3α,17β-diol	0	0	1	3	10	**87**
(16,16,17-$^2H_3$)-5α-Androstane-3β,17β-diol	0	1	9	**90**		
(2,2,4,4-$^2H_4$)-Norandrosterone	0	0	1	6	**93**	

Table 1: Isotopic composition of deuterated steroid RMs

- Isotopic purity was determined by SIM analysis (mean of three replicates) of the bis-TMS derivatives using a HP 6890/5973 instrument. Results are uncorrected for small and in part cancelling contributions due to $[M-H]^+$, $[M-2H]^+$ and ^{13}C isotope peaks of partially labelled material.

Steroid Category	*Parent*	*Glucuronide*	*Sulphate*
• **Deuterated endogenous steroids**			
d_3-Testosterone	▲	▲	▲
d_3-Epitestosterone	▲	●	✚
d_3-5α-Dihydrotestosterone	▲	✚	✚
d_4-Androsterone	▲	✚	✚
d_3-5β-Dihydrotestosterone	✚		✚
d_5-Etiocholanolone	▲	●	✚
d_3-5α-Androstane-3α,17β -diol	▲		
d_3-5α-Androstane-3β,17β -diol	✚		
d_5-5β-Androstane-3α,17β -diol	▲		
d_5-5β-Androstane-3β,17β -diol	✚		
• **Endogenous steroids**			
Testosterone	✚	✚	✚
Epitestosterone	✚	✚	✚
5α-Dihydrotestosterone	✚	●	✚
Androsterone	✚	✚	✚
5β-Dihydrotestosterone	✚		✚
Etiocholanolone	✚	●	✚
5α-Androstane-3α,17β-diol	✚	●	
5α-Androstane-3β,17β-diol	✚		
5β-Androstane-3α,17β-diol	✚	●	
5β-Androstane-3β,17β-diol	✚		

▲ = Production as RM completed and reviewed
✚ = Synthesis and characterisation complete
● = In progress or planned

Table 2: July 1999 status - Endogenous Anabolic Steroid RM Production

- **Synthetic anabolic steroid metabolites**

	Metabolite of	*Parent*	*Glucuronide*
19-Norandrosterone	**Nandrolone**	▲	✚
d_4-19-Norandrosterone	"	▲	●
19-Noretiocholanolone	"	▲	✚
d_4-19-Noretiocholanolone	"	●	●
17α-Ethyl-5α-estrane-3α,17β-diol	**Ethandrolone**	▲	●
17α-Ethyl-5β-estrane-3α,17β-diol	"	▲	●
7α,17α-Dimethyl-5β-androstane-3α,17β-diol	**Bolasterone**	●	●
7β,17α-Dimethyl-5β-androstane-3α,17β-diol	**Calusterone**	●	●
2-Hydroxymethyl-17α-methylandrosta-1,4-diene-11α,17β-diol-3-one	**Formebolone**	●	
17α-Methyl-5α-androstane-3α,17β-diol	**Methyltestosterone**	▲	●
17α-Methyl-5β-androstane-3α,17β-diol	"	▲	●
17-Epioxandrolone	**Oxandrolone**	●	
17-Epimethandienone	**Methandienone**	▲	
6β-Hydroxymetandienone	"	✚	
17,17-Dimethyl-18-norandrostatrien-3-one	"	✚	
3'-Hydroxystanozolol	**Stanozolol**	✚	●
4β-Hydroxystanozolol	"	●	●
16β-Hydroxystanozolol	"	●	●
16β-Hydroxyfurazabol	**Furazabol**	✚	●
1-Methylene-5α-androstan-3α-ol-17-one	**Metenolone**	✚	●
1α-Methyl-5α-androstan-3α-ol-17-one	**Mesterolone**	▲	●
1α-Methyl-5α-androstan-3α,17β-diol	"	▲	●
5β-Androst-1-en-17β-ol-3-one	**Boldenone**	▲	●
17α-Boldenone	"	✚	
d_3-Boldenone	"	✚	
2α-Methyl-5α-androstane-3α-ol-17-one	**Drostanolone**	▲	●
Clostebol		●	
4-Chloroandrost-4-en-3-α-ol-17-one	**Clostebol**	▲	
6β-Hydroxy-1,2-dehydro-4-chloro-17α-methyltestosterone	**Dehydrochloromethyl testosterone**	●	
4-Chloro-3α,6β,17β-trihydroxy-17α-methyl-5β-androst-1-ene-16-one	"	●	
9α-Fluoro-17,17-dimethyl-18-norandrostadiene-11β-ol-3-one	**Fluoxymesterone**	✚	●
6β-Hydroxyfluoxymesterone	"	●	●

▲ = Production as RM completed and reviewed
✚ = Synthesis and characterisation complete
● = In progress or planned

Table 3: July 1999 status - Synthetic Anabolic Steroid RM Production